



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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In re the Application of Timothy P. Croughan

Examiner Kruse, D.

Serial No. 09/830,194

International Filing Date 05 November 1999

Group 1638

35 U.S.C. § 371 Date 23 April 2001

For: Herbicide Resistant Rice

Atty. File 98A9-US Croughan

Commissioner for Patents  
Box Non-Fee Amendment  
Washington, D.C. 20231

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AFFIDAVIT OF INVENTOR TIMOTHY P. CROUGHAN

STATE OF LOUISIANA

PARISH OF ACADIA

Timothy P. Croughan, being duly sworn, deposes and says:

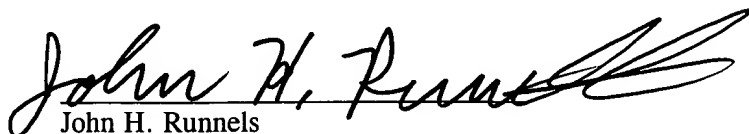
1.

I am the inventor of the above-identified patent application. I make this Affidavit in support of that application.

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CERTIFICATE

I hereby certify that this Affidavit of Inventor Timothy P. Croughan is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Box Non-Fee Amendment, Washington, D.C. 20231 on December 20, 2002.



John H. Runnels

Registration No. 33,451

December 20, 2002

2.

All experiments reported in the specification, and all experiments described in the present Affidavit were conducted by me, or were conducted under my supervision.

3.

The procedures used to assay the activity of the acetohydroxyacid synthases from various rice lines as reported below were substantially as described in B.K. Singh *et al.*, "Assay of Acetohydroxyacid Synthase," *Analytical Biochemistry*, vol. 171, pp. 173-179 (1988), except as noted. In the first paragraph of Singh's "Materials and Methods," instead of corn suspension culture cells, shoot tissues from greenhouse-grown rice seedlings at the 3-4 leaf stage of development, or rice suspension culture cells were used. For shoot tissues, 40.0 or 50.0 grams (fresh weight) of tissue were extracted in the same manner for each of the breeding lines; for Cypress suspension cells, 16.0 grams of cells were used, harvested eight days after subculture. At the suggestion of the first author, B.K. Singh (personal communication), the desalting step mentioned at the bottom of Singh's first column under "Materials and Methods" was eliminated. Pursuit™ herbicide (imazethapyr) or Arsenal™ herbicide (imazapyr) was included in the "standard reaction mixture" for the AHAS assay in various concentrations. Colorimetric absorbance was measured at 520 nm. Checks were made of direct acetoin formation during the enzyme assay.

4.

Nine rice lines have been assayed in this manner: the non-resistant Cypress line (which was the "parental" line for some of the herbicide-resistant mutant lines, including PTA-904), the resistant line PTA-904, and seven other resistant lines for which results are

not reported here, because those lines pertain primarily to Claims that I understand are being cancelled from the present application in response to a restriction requirement. Some assays were conducted at different times, and assays at some herbicide concentrations were repeated. In the modified Singh assay for total AHAS activity, using crude enzyme extract, in the absence of herbicide, line PTA-904 actually expressed lower total AHAS activity than did the non-resistant Cypress line. Following treatment with the herbicides Pursuit (imazethapyr) or Arsenal (imazapyr), the reduction in AHAS activity for the nonresistant Cypress line was substantial. The AHAS enzyme from line PTA-904 expressed strong resistance to both imazethapyr and imazapyr, while the nonresistant Cypress line was sensitive to both herbicides. Results are shown in Table 1. In Table 1, the first column ("No herbicide") is reported as absorbance at 520 nm. All other entries in a given row (i.e., for a given line of rice) are reported as a percentage of the absorbance for the same rice line in the absence of herbicide.

**Table 1 -- Total AHAS Activity, Crude Enzyme Extracts,  
measured as absorbance at 520 nm**

	No herbicide	Imazethapyr				Imazapyr		
		50 $\mu$ M	100 $\mu$ M (first replicate)	100 $\mu$ M (second replicate)	1000 $\mu$ M	50 $\mu$ M	100 $\mu$ M	1000 $\mu$ M
Cypress	0.766	63 %	54 %	58 %	56 %	63 %	57 %	45 %
ATCC PTA-904 (PWC16)	0.713	95 %	83 %	92 %	78 %	90 %	83 %	76 %

5.

The results shown in Table 1 clearly show that the resistant line PTA-904 contains a resistant mutant AHAS enzyme. The lowest herbicide concentrations tested, 50  $\mu$ M of imazethapyr or imazapyr, reduced the activity of the non-resistant line's AHAS to about 63% of control -- a reduction in activity that is lethal to plants in the field. By contrast, the resistant PTA-904 line had AHAS activities of 95% or 90% of control, respectively, at these herbicide concentrations. Even at the highest herbicide concentration tested, 1000  $\mu$ M, enzyme activities in the resistant plants were 78% and 76%, respectively; while the enzyme activities for the nonresistant Cypress line were substantially lower, 56% and 45%, respectively. Put differently, the resistant PTA-904 line showed higher AHAS activity at the extremely high herbicide concentration of 1000  $\mu$ M than the AHAS activity of the non-resistant line at the lowest herbicide rate tested, 50  $\mu$ M.

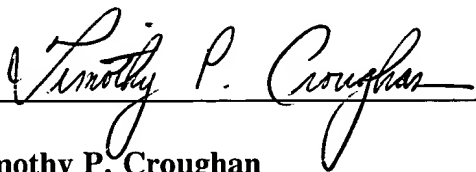
6.

The results given in Table 1 clearly demonstrate that the herbicide resistance characteristics of PTA-904 were due to a resistant mutant AHAS enzyme. By contrast, as explained more fully in my U.S. Patent 5,736,629, Col. 6, lines 7-64, the herbicide resistance of ATCC 75295, a resistant rice line that was disclosed in my U.S. Patent 5,545,822, is due to an unknown mechanism. As reported more fully in my U.S. Patent 5,736,629, this resistance mechanism is affirmatively known not to be the result of a

mutant AHAS enzyme. Thus the development of line PTA-904 was neither a straightforward nor a predictable extension of the earlier work that led to ATCC 75295.

7.

All statements made in this Affidavit of my own knowledge are true. All statements made in this Affidavit on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified patent application or any patent issuing from that application.

  
\_\_\_\_\_  
Timothy P. Croughan

**SWORN TO AND SUBSCRIBED**

before me this 18th day  
of December, 2002.

  
\_\_\_\_\_  
NOTARY PUBLIC

MARY KAY Hetzel  
[Printed Name of Notary]

**My Commission Expires at Death**

[SEAL]